

Appl. No. 10/519,390
Amdt. dated May 28, 2007
Reply to Office Action mailed November 28, 2006

Amendments to the Specification:

Please replace the paragraph appearing at page 35, lines 3-9 with the following amended paragraph:

2 µg of mRNA purified as above and 1 µl of oligo dT30 primer (10 µM, ~~Promega~~ PROMEGA, USA) were mixed and heated at 70° C for 2 minutes and then it was immediately cooled on ice for 2 minutes. After that, this reaction mixture was added with 200 U M-MLV reverse transcriptase (~~Promega~~ PROMEGA, USA), 10 µl of 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 nM DTT), 1 µl of dNTP (10 mM dATP, 10 mM dTTP, 10 mM dGTP, 10 mM dCTP) and DEPC-treated 3 °C water was added to make the total volume of 50 µl. After mixing gently, the reaction mixture was incubated at 42° C for 60 minutes.

Please replace the paragraph appearing at page 35, lines 10-17 with the following amended paragraph:

To amplify cDNA coding wild type TPO, the first strand cDNA as template, primer 1 and primer 2 (Table 1) were added into a PCR tube including 2 U of pfu DNA polymerase (~~Stratagene~~ STRATAGENE, USA), 10 µl of 10x reaction buffer, 1% ~~Triton~~ TRITON X-100, 1 mg/ml BSA, 3 µl of primer 1 (10 µM), 3 µl of primer 2 (10 µM), 2 µl of dNTP (10 mM dATP, 10 mM dTTP, 10 mM dGTP, 10 mM dCTP), and distilled water was added to make the total volume of 100 µl. The PCR reaction condition was as follows; 1 cycle at 95° C for 3 minutes, and then 30 cycles at 95° C for 30 seconds, at 52° C for 1 minute, and at 72° C for 1.5 minutes, and finally 1 cycle at 72° C for 10 minutes to make PCR product with completely blunt end.

Please replace the paragraph appearing at page 35, lines 18-22 with the following amended paragraph:

The PCR product obtained was separated in 0.8% agarose gel (BMA, USA) and was purified with Qiaex II gel extraction kit (~~Qiagen~~ QIAGEN, USA). After the isolated DNA was mixed with 15 U of EcoRI 10 U of NotI, 3 µl of 10x reaction buffer and 3 °C distilled water was

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added to make the total volume of 30 μ l, DNA was restricted by incubation at 37° C for 2 hours. The PCR product was separated in 0.8% agarose gel and was purified with Qiaex II gel extraction kit.

Please replace the paragraph appearing at page 35, lines 23-26 with the following amended paragraph:

After 5 μ g of pBluescript PBLUESCRIPT KS II(+) vector was mixed with 15 U of EcoRI, 10 U of NotI, 3 μ l of 10x reaction buffer and 3° C distilled water was added to make the total volume of 30 μ l, DNA was restricted by incubation at 37° C for 2 hours. The restricted pBluescript PBLUESCRIPT KS II(+) vector was separated in 0.8% agarose gel and was purified with Qiaex II gel extraction kit.

Please replace the paragraph appearing at page 35, line 27 through page 36, line 7 with the following amended paragraph:

100 ng of the digested pBluescript KS II(+) vector was ligated with 20 ng of the PCR product which was digested with same enzymes. This ligation mixture was incubated at 16° C. water bath for 16 hours, thus producing a recombinant vector comprising cDNA coding wild type TPO. Then, it was transformed into a E.coli Top10(~~Invitrogen~~ INVITROGEN, USA) which was made to a competent cell by rubidium chloride method. The transformed bacteria was cultured on LB agar plate containing 50 μ g/ml of ampicillin (~~Sigma~~ SIGMA, USA). After overnight incubation, colonies were transferred into tubes with 3 ml of LB medium containing 50 μ g/ml ampicillin and then they were cultured at 37° C for 16 hours. Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of EcoRI/NotI was used to detect inclusion of cloned gene in the plasmid.

Please replace the paragraph appearing at page 36, lines 12-17 with the following amended paragraph:

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The first strand cDNA as template, primer 11 and primer 12 (Table2) were used for PCR amplification of DNA coding wild type EPO. The PCR product and cloning vector, pBluescript KS II(+) were digested with both EcoRI and BamHI endonucleases. The digested PCR product and cloning vector were ligated and transformed into competent cell, E.coli Top10(~~Invitrogen~~ INVITROGEN, USA). Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of EcoRI/BamHI was used to detect existence of cloned gene in the plasmid.

Please replace the paragraph appearing at page 36, lines 23-27 with the following amended paragraph:

Leukocytes from healthy people were used for the mRNA extraction, and primers 21 and 22 (Table 3) were used for PCR amplification of cDNA coding wild type G-CSF. Both the PCR product and cloning vector, pBluescript KS II(+) were digested with SmaI and EcoRI endonuclease. The digested PCR product and cloning vector were ligated and transformed into competent cell, E.coli Top10 (~~Invitrogen~~ INVITROGEN, USA). Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of SmaI/EcoRI was used to detect existence of cloned gene in the plasmid.

Please replace the paragraph appearing at page 43, line 23 through page 44, line 13 with the following amended paragraph:

The cells transfected with plasmid containing cDNA coding TPO-wild type or muteins were analyzed on their protein expression level by using ELISA assay. An goat anti-human TPO polyclonal antibody (R&D, U.S.A) diluted to 10 µg/ml with coating buffer [0.1M Sodium bicarbonate, (pH 9.6)] was added into each wells of 96 well plate (Falcon, USA) up to 100 µl per well and incubated for 1 hour at room temperature. The plate was washed with 0.1% Tween-20 in 1x PBS (PBS three times. After washing, the plate was incubated with 200 µl of blocking buffer (1% FBS, 5% sucrose, 0.05% sodium azide) for 1 hour at room temperature and then washed three times with PBST. The cultured supernatants (including the transfected cells) and

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dilution buffer[0.1% BSA, 0.05% Tween-20, 1xPBS] were mixed with serial dilutions. 25 ng/ml of recombinant human TPO [~~Calbiochem~~ CALBIOCHEM, USA] as a positive control and untransfected CHO-K1 cultured supernatants as a negative control were equally diluted. These controls and samples were incubated for 1 hr at room temperature. Then, the plate was washed with PBST three times. A biotinylated goat anti-human TPO antibody (R&D, USA) diluted to 0.2 µg/ml with dilution buffer was added to the 96 well plate up to 100 µl per well and incubated for 1 hr at room temperature. The plate was washed with PBST three times. Streptavidin-HRP (R&D, USA) diluted to 1:200 in dilution buffer was added 100 µl per well to the 96 well plate and incubated for 1 hr at room temperature. After 1 hour, the plates was washed three times with PBST, and then coloring reaction was performed by using TMB microwell peroxidase substrate system (KPL, USA) and OD was read at 630 nm with microplate reader[BIO-RAD, Model 550].

Please replace the paragraph appearing at page 49, lines 4-11 with the following amended paragraph:

TPO receptor-Ig fusion protein was purified from culture supernatant of CHO cell transfected with recombinant expression vector carrying gene coding for TPO receptor-Fc fusion protein by using Protein A Sepharose-4B column (Pharmacia PHARMACIA, Sweden). The purified fusion protein diluted to 10 µg/ml with coating buffer [0.1M Sodium bicarbonate, (pH 9.6)] was added into each wells of 96 well plate (Falcon, USA) up to 100 µl per well and incubated for 1 hour at room temperature. The plate was washed with 0.1% Tween-20 in 1X PBS [PBST] three times. After washing, the plate was incubated with 200 µl of blocking buffer (1% FBS, 5% sucrose, 0.05% sodium azide) for 1 hour at room temperature and then washed three times with PBST.

Please replace the paragraph appearing at page 49, lines 12-23 with the following amended paragraph:

After washing, culture supernatants consisting of four TPO muteins and one TPO wild type, respectively were diluted serially with dilution buffer [0.1% BSA, 0.05% Tween-20, 1x

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PBS] and was added to 96 well plate coated with the TPO receptor-Fc fusion protein and incubated for 1 hr. The washing was repeated three times with PBST. A recombinant human TPO [~~Calbiochem~~ CALBIOCHEM, USA] as a positive control and untransfected CHO-K1 cultured supernatants as a negative control were equally diluted. The plates were washed with PBST three times. A biotinylated goat anti-human TPO antibody (R&D, USA) diluted to 0.2 µg/ml in dilution buffer was added to the 96 well plate to 100 µl per well and incubated for 1 hr at room temperature. The plate was washed with PBST three times. Streptavidin-HRP (R&D, USA) diluted to 1:200 in dilution buffer was added 100 µl per well to 96 well plate and incubated for 1 hr at room temperature. The plate was washed three times with PBST after 1 hour. Coloring reaction was performed using TMB microwell peroxidase substrate system (KPL, USA) and O.D was read at 630 nm with microplate reader [BIO-RAD, Model 550].

Please replace the paragraph appearing at page 52, lines 5-11 with the following amended paragraph:

TF-1/c-Mpl cell line was established by transfecting cDNA coding c-Mpl into TF-1 cell. Expression of c-Mpl was verified by using FACS analysis. The 1×10^6 /ml of the TF-1/c-Mpl cells was washed with PBS buffer and purified c-Mpl mouse anti-human monoclonal antibody (BD PharMingen, USA) was incubated with the TF-1/c-Mpl cells. And then FTC-conjugated anti-mouse IgG (whole molecule; Sigma SIGMA, USA) was added to verify expression of c-Mpl on surface of the TF-1/c-Mpl cells. As a result, graph of the TF-1/c-Mpl cell was shifted rightward from that of TF-1 cells. This result showed that c-Mpl, TPO receptor, was expressed on the TF-1/c-Mpl cell.

Please replace the paragraph appearing at page 52, lines 14-20 with the following amended paragraph:

The 1×10^6 /ml of TF-1/c-Mpl cell was suspended in PBS buffer and TPO wild type and -[F141V] was added to the suspension and incubated at 4°C for 30-60 minutes, respectively.

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Biotinylated goat anti-human TPO polyclonal antibody (R&D, USA) was added to the cells above and incubated at 4°C for 30-60 minutes. Streptavidin-FITC (~~Sigma~~ SIGMA, USA) was added to the cells above and incubated at 4°C for 30-60 minutes. The cells were washed twice with PBS buffer to remove non-reacted Streptavidin-FITC. The cells were suspended in PBS buffer and flow cytometric analysis was performed at 488 nm using EXCALIBUR (BD, U.S.A).

Please replace the paragraph appearing at page 53, lines 8-170 with the following amended paragraph:

To investigate differences of cell proliferation and biological activities between TPO-wild type and muteins, TF-1/c-Mpl cell line produced above was used. TF-1c-Mpl cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 1 ng/ml GM-CSF at 37° C, 5% CO₂. 0.4, 1, 5, 10, 20, 40, 75 ng/ml of each of TPO-wild type and muteins in RPMI-1640 were seeded in 96-well tissue-culture plates (FALCON, USA). 1x10⁴ cells of the TF-1c-Mpl cells in RPMI-1640 containing 10% fetal bovine serum was added to each well of the 96-well plate. After 4 days cultivation at 37° C, 5% CO₂, 20 µl of MTS solution [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS] and the phenazine ethosulfate (PES; ~~promega~~ PROMEGA) was added and incubated for 4 hours. O.D. was measured with microplate reader (BIO-RAD Model 550) at 490 nm.